(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 404 098 A1**

(12) # EUROPEAN PATENT APPLICATION
published in accordance with Art. 153(4) EPC

(43) Date of publication:
21.11.2018 Bulletin 2018/47

(21) Application number: **17738392.4**

(22) Date of filing: **10.01.2017**

(51) Int Cl.:
C12N 15/09 (2006.01) ***C12N 1/19*** (2006.01)
C12P 21/00 (2006.01)

(86) International application number:
PCT/JP2017/000497

(87) International publication number:
WO 2017/122638 (20.07.2017 Gazette 2017/29)

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME
Designated Validation States:
MA MD

(30) Priority: **12.01.2016 JP 2016003606**

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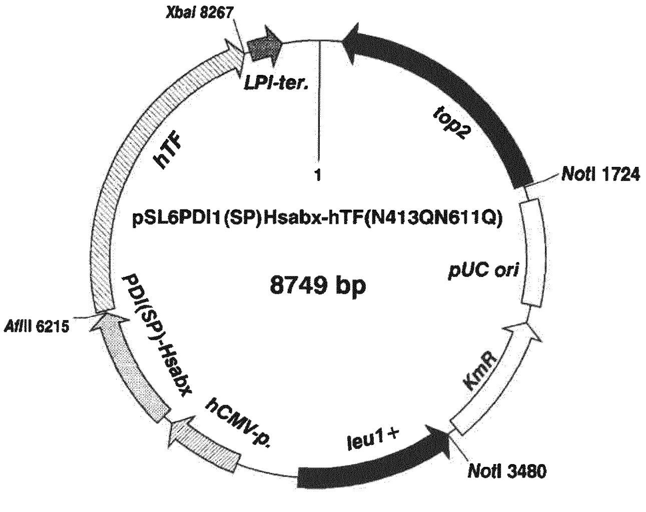
(54) # TRANSFORMANT, AND TRANSFERRIN MANUFACTURING METHOD

(57) To provide a method for producing transferrin by secretion with high productivity using a transformant of *Schizosaccharomyces pombe,* and a transformant suitable for the method.

A transformant using *Schizosaccharomyces pombe* as a host, comprising a transferrin gene and a secretion signal peptide gene expressing transferrin to which a secretion signal peptide which functions in the host binds, located in an upstream region of the transferrin gene, and having Gas2 gene endogenous to the host deleted or inactivated; and a method for producing transferrin, which comprises culturing the transformant in a liquid medium and obtaining transferrin from the liquid medium.

Fig. 1



Printed by Jouve, 75001 PARIS (FR)

Description

TECHNICAL FIELD

[0001] The present invention relates to a method for producing transferrin by using a transformant from *Schizosaccharomyces pombe* (hereinafter referred to as "*S. pombe*") as a host and having a transferrin gene integrated thereinto.

BACKGROUND ART

[0002] Transferrin (hereinafter sometimes referred to as "TF") is one of glycoproteins which bind to Fe^{3+} ions and is deeply involved in iron metabolism. Particularly human serum transferrin (hereinafter sometimes referred to as "human transferrin" or "hTF") belongs to a family of iron-binding proteins in a human body, is involved in iron transport/metabolism in the body and is used as additives to be used for a medium for animal cell culture, a pharmaceutical preparation or a DDS carrier. TF is an about 80 kDa glycoprotein having two domains named N-lobe and C-lobe, and it is synthesized by translation as an immature protein having a secretion signal peptide at its N-terminal and is then secreted out of the cell as a mature glycoprotein having the secretion signal peptide cleaved.

[0003] As the most common method for TF production, a method for producing a full-length recombinant protein by using a cell line of mammal cells such as baby hamster kidney cells (BHK cells) has been known. Further, a method of using budding yeast *S. pombe* has been known. *S. pombe* has, as different from budding yeast *Saccharomyces cerevisiae,* cell division and transcription forms close to those of human cells and contains no substance which has adverse effects on the body. Accordingly, the method for producing TF full-length recombinant protein employing *S. pombe* is excellent as a method for producing TF to be administered to human, such as a pharmaceutical preparation. For example, Patent Document 1 discloses a method such that a transformant using *S. pombe* as a host and having a *hTF* gene introduced is cultured in a liquid medium containing casamino acids to efficiently produce hTF, which is made to be secreted to the liquid medium and recovered.

[0004] On the other hand, in a heterologous protein production system employing an eukaryotic microorganism such as a yeast, in order to improve the efficiency for production of the desired heterologous protein, it has been known to use an improved host having a part of or the entire genome portion of a host unnecessary or disadvantageous for heterologous protein production eliminated or inactivated. For example, Patent Document 2 discloses that use of an improved host having at least one gene selected from genes encoding a specific protease (protease gene group) of *S. pombe* eliminated or inactivated, improves the efficiency for production of a heterologous protein.

[0005] Further, hTF protein can be secreted and produced by production in a state where a secretion signal (endoplasmic reticulum targeting signal) peptide is added to the N-terminal of hTF protein. For example, by culturing a transformant of *S. pombe* which has introduced a structural gene encoding a fusion protein having a secretion signal peptide recognized by *S. pombe,* such as a polypeptide derived from secretion signal of a precursor of a mating pheromone (P-factor) relating to mating of *S. pombe,* fused to the N-terminal of hTF protein, from the produced fusion protein, secretion signal peptide is removed in the Golgi apparatus or the endoplasmic reticulum, and hTF protein is secreted into the medium. For example, Patent Document 3 discloses that by expressing, at the N-terminal side of the desired foreign protein, a partial protein composed of a secretion signal peptide of PDI1 (protein disulfide isomerase 1) which is a protein having a molecular chaperone function and localized in the endoplasmic reticulum, an a-domain, a b-domain and an x-domain, or a partial protein composed of an endoplasmic reticulum targeting signal peptide of PDI1, an a-domain, a b-domain, a b'-domain and an x-domain, in a fused state, the amount of secretory production of the foreign protein by *S. pombe* can be increased. PDI1 comprises, from the N-terminal, an ER targeting signal, a-domain, b-domain, b'-domain, x-domain, a'-domain and c-domain containing an ER retention signal (ADEL), in this order. Each of the a-domain and the a'-domain has one active site (CGHC) for molecular chaperone activity. All the four domains a, b, b' and a' form a thioredoxin fold.

PRIOR ART DOCUMENTS

PATENT DOCUMENTS

[0006]

Patent Document 1: JP-A-2011-125281
Patent Document 2: WO2007/015470
Patent Document 3: WO2013/111754

DISCLOSURE OF INVENTION

TECHNICAL FIELD

[0007] The object of the present invention is to provide a method for producing TF by secretion with high productivity using a transformant of *S. pombe,* and a transformant suitable for the method.

SOLUTION TO PROBLEM

[0008] The transformant of the present invention is a transformant using *S. pombe* as a host, comprising a *TF* gene and a secretion signal peptide gene expressing TF to which a secretion signal peptide which functions in the host binds, located in an upstream region of the *TF* gene, and having *Gas2* gene endogenous to the host deleted or inactivated.

[0009] The transformant is preferably such that the *TF* gene is a gene encoding human transferrin.

[0010] Further, the transformant is preferably such that the *TF* gene is a mutant *TF* gene having a mutation introduced to a gene encoding natural mammal TF, and the mutant *TF* gene is a gene encoding mutant TF having at least one aspartic residue which is an N-linked glycosylated portion of natural TF deleted or substituted with another amino acid residue.

[0011] Further, the transformant is preferably such that at least one protease gene endogenous to the host is deleted or inactivated. The protease gene is preferably a gene selected from the group consisting of a metalloproteinase gene group, a serine protease gene group, a cysteine protease gene group and an aspartic protease gene group. The protease gene to be deleted or inactivated is preferably at least one gene selected from the group consisting of *psp3* gene, *isp6* gene, *ppp53* gene, *ppp16* gene, *ppp22* gene, *sxa2* gene, *ppp80* gene and *ppp20* gene.

[0012] The transformant is preferably such that the *TF* gene is located downstream of a gene encoding a secretory carrier protein containing a secretion signal peptide, directly or indirectly, and more preferably such that the gene encoding a secretory carrier protein is a fusion gene of a gene encoding a secretion signal peptide portion of host PDI1, a gene encoding an ab-domain portion of human PDI1, and a gene encoding an x-domain portion of host PDI1.

[0013] The method for producing TF of the present invention comprises culturing the transformant of the present invention in a liquid medium and obtaining transferrin from the liquid medium.

[0014] The method for producing TF is preferably such that the transformant is cultured in a liquid medium having a pH of from 5.5 to 6.5, preferably such that the transformant is cultured in a liquid medium containing adenine, and also preferably such that the transformant is cultured until the cell-density (OD_{660}) reaches 100 or higher, and then transferrin is obtained from the liquid medium.

[0015] The method for producing a transformant of the present invention comprises integrating into *S. pombe* as a host, a secretion signal peptide gene which functions in the host, and a *TF* gene located downstream of the secretion signal peptide gene, and deleting or inactivating Gas2 gene endogenous to the host.

ADVANTAGEOUS EFFECTS OF INVENTION

[0016] By culturing the transformant of the present invention, TF can be produced efficiently.

[0017] Further, according to the method for producing TF of the present invention, TF can be produced by secretion with high productivity using *S. pombe.*

BRIEF DESCRIPTION OF DRAWINGS

[0018]

Fig. 1 is a diagram illustrating the structure of secretory expression vector pSL6PDI1(SP)Hsabx-hTF(N413QN611Q).
Fig. 2 is a CBB stained image of culture broths of A8-hTF(1) strain and A8-gas2Δ-hTF(1) strain after SDS-PAGE.
Fig. 3 is a graph illustrating changes with time of the secretion amounts of hTF(N413Q/N611Q) protein from A8-hTF(1) strain and A8-gas2Δ-hTF(1) strain.
Fig. 4 is CBB stained images of fractions of hTF(N413Q/N611Q) protein in culture supernatants of A8-hTF(1) strain and A8-gas2Δ-hTF(1) strain, purified by DEAE chromatography, after SDS-PAGE.

DESCRIPTION OF EMBODIMENTS

[*TF* gene, secretion signal peptide gene]

[0019] In the present invention, "*TF* gene" means a structural gene encoding mature TF protein. Further, the mature

TF protein will be hereinafter referred to as "TF protein".

[0020] In cells of a species to which the *TF* gene is endogenous, a fusion protein having a protein (secretory carrier protein) containing a secretion signal peptide and TF protein linked to each other, is expressed, and from the fusion protein, the secretory carrier protein is cleaved out in the endoplasmic reticulum or the Golgi apparatus, and the TF protein is secreted out of the cells.

[0021] On the other hand, since *TF* gene is exogenous to *S. pombe* as a host in the present invention, even when a fusion protein having a secretory carrier protein and TF protein linked to each other is expressed in the host, the secretory carrier protein is not necessarily cleaved out in the hot cells. Accordingly, as the secretion signal peptide or the secretory carrier protein in the present invention is preferably a secretion signal peptide or a secretory carrier protein other than one endogenous to the host at its N-terminal side of TF protein, which sufficiently functions in the host. In the present invention, the term (a secretion signal peptide which) "functions in the host" means having a function to secrete the fusion protein expressed in the host out of the host cells as the TF protein.

[0022] Further, in the present invention, the TF protein and the secretion signal peptide or the secretory carrier protein may be directly linked to each other, or may be indirectly linked to each other by a linker composed of a peptide comprising one to several tens amino acid residues.

[Transformant]

[0023] The transformant of the present invention is a transformant using *S. pombe* as a host, comprising a *TF* gene and a secretion signal peptide gene expressing TF protein to which a secretion signal peptide which functions in the host binds, located in an upstream region of the *TF* gene, and having *Gas2* gene endogenous to the host deleted or inactivated.

[0024] The TF protein encoded by the *TF* gene which the transformant of the present invention has, may be a natural TF protein derived from any species (TF protein encoded by a *TF* gene present in the chromosome of a wild type organism) or may be a modified protein of a natural TF protein. The modified protein may, for example, be a polypeptide comprising an amino acid sequence of a natural TF protein, in which one or several amino acids are substituted, added or deleted, and having a TF function. A polypeptide comprising an amino acid sequence having an identity to the amino acid sequence of a natural TF protein of at least 80%, preferably at least 85%, more preferably at least 90%, further preferably at least 95%, and having a TF function, may be mentioned. The TF function means a function to bind to Fe^{3+} ions and to bind to the TF receptor on the cell surface to be incorporated into the cell. The TF protein encoded by the *TF* gene which the transformant of the present invention has, is preferably a natural TF protein derived from a mammal such as human, monkey, mouse, rat, rabbit, cattle, horse, dog, cat, etc., or a modified protein thereof, particularly preferably hTF or a modified protein thereof.

[0025] In a case where the TF protein encoded by the *TF* gene which the transformant of the present invention has, is a modified protein of a natural TF protein, the modification is, for example, preferably point mutation such that at least one aspartic acid residue as the N-linked glycosylated portion after translation, is deleted or substituted by another amino acid residue. In a case where a protein having a N-linked glycosylated portion is produced by secretion, the resulting recombinant protein is modified with a high mannose sugar chain having non-uniform length, and the uniformity or the quality may thereby be decreased.

[0026] In a case where the *TF* gene which the transformant of the present invention has, is a modified *TF* gene encoding a modified TF protein mutated to delete the N-linked glycosylated portion, by culturing such a transformant, a recombinant TF protein which has a stable TF function and which has a uniform quality can be produced by secretion. For example, such a modified *TF* gene is preferably a modified *TF* gene encoding a modified TF protein in which at least one aspartic acid residue as an N-linked glycosylated portion of the two N-linked glycosylated portions (432nd and 630th aspartic acid residues) of a natural hTF protein, is deleted or substituted with an amino acid residue other than an aspartic acid residue, more preferably a modified *TF* gene encoding a modified TF protein in which the two N-linked glycosylated portions are substituted with an amino acid residue other than an aspartic acid residue, further preferably a modified *TF* gene (SEQ ID NO: 2) encoding a mutant TF protein in which the two N-linked glycosylated portions are substituted with glutamine residue.

[0027] The secretion signal peptide gene which the transformant of the present invention has is a structural gene encoding a secretion signal peptide which functions in the transformant cell. The secretion signal peptide gene may be a part of a structural gene encoding a secretory carrier protein. That is, the structural gene encoding a secretory carrier protein may have a structural gene portion encoding a secretory carrier protein other than the secretion signal peptide gene portion, downstream the secretion signal peptide gene. The secretion signal peptide may be any peptide which functions in *S. pombe* and may, for example, be ones disclosed in WO1996/23890 such as a secretion signal peptide of a secretory protein endogenous to *S. pombe,* or P3 secretion signal (34th amino acid residue), and is preferably a secretion signal peptide of *S. pombe* PDI1.

[0028] The secretory carrier protein which functions in *S. pombe* is preferably a PDI1 full-length protein containing a secretion signal peptide, a PDI1 partial protein, or a protein having such a modified protein fused. Particularly preferred is a protein containing a-domain of PDI1 or a protein containing a'-protein of PDI1, more preferably a protein containing at least one of a-domain and a'-domain and at least one of b-domain and b'-domain of PDI1, further preferably a protein containing at least one of a-domain and a'-domain, at least one of b-domain and b'-domain and x-domain of PDI1 (see Patent Document 3). All the respective domains of PDI1 contained in the protein fused downstream the secretion signal peptide may be derived from a single species, or they may be a combination of domains derived from two or more species.

[0029] In the transformant of the present invention, in order for more increased TF secretory production capability, the secretory carrier protein is preferably a fused gene having, in order from the upstream side, a secretion signal peptide gene of *S. pombe* PDI1 and a gene encoding abx-domain portion of human PDI1 or a gene encoding abb'x-domain portion of human PDI1 directly or indirectly linked, or a fusion protein having a secretion signal peptide gene of *S. pombe* PDI1, a gene encoding ab-domain portion of human PDI1 or a gene encoding abb'-domain portion of human PDI1, and a gene encoding x-domain portion of *S. pombe* PDI1 directly or indirectly linked.

[0030] *TF* gene is exogenous to *S. pombe.* Accordingly, the transformant of the present invention is obtained by biogenetically introducing a *TF* gene derived from an organism other than *S. pombe,* into *S. pombe.* The nucleotide sequence of the *TF* gene which the transformant of the present invention has may be the gene sequence of the species from which the TF protein is derived as it is, or the gene sequence may be modified by changing its codons to ones frequently used in *S. pombe.*

[0031] The copy number of a gene having the secretion signal peptide gene and the *TF* gene directly or indirectly linked to each other of the transformant (hereinafter referred to as *SP-TF* gene) may be one or two or more. The *SP-TF* gene may be introduced as the plasmid as an extrachromosomal gene in *S. pombe,* or may be introduced into the plasmid of *S. pombe.* By introducing a foreign gene to the chromosome, a transformant excellent in the passage stability can be obtained.

[0032] Regarding the gene recombination method using yeast as a host, for more stably and efficiently expressing heterologous proteins, various expression systems, particularly an expression vector, a secretion signal, a gene introduction expression vector, etc. are developed, and for production of the transformant of the present invention, they are widely applicable. For example, as an expression system using *S. pombe* as a host, Japanese Patent No. 2776085, JP-A-H07-163373, JP-A-H10-215867, JP-A-H10-215867, JP-A-H11-192094, JP-A-H11-192094, JP-A-2000-262284, WO96/023890 etc. have been known, and for production of the transformant of the present invention, such expression systems are widely applicable.

[0033] Introduction of the *SP-TF* gene into *S. pombe* as a host is carried out, for example, by introducing an expression cassette containing the *SP-TF* gene, and a promoter and a terminate which function in *S. pombe* as it is into the host cell, or by introducing a vector having such a cassette integrated into the host cell. The expression cassette may contain at least one of a 5'-untranslation region and a 3'-untranslation region, and may contain an auxotrophic complementation marker such as *ura4* gene.

[0034] As the promoter which functions in *S. pombe,* a promoter endogenous to *S. pombe,* such as an alcohol dehydrogenase gene promoter, a *nmt1* gene promoter, a fructose 1,6-bisphosphatase gene promoter, an invertase gene promoter (WO99/23223) or a heat shock protein gene promoter (WO2007/26617, WO2014/030644), or a promoter derived from an animal cell virus such as a hCMV promoter or a SV40 promoter may, for example, be mentioned. As the terminator which functions in *S. pombe,* a LPI (human lipocortin I) terminator may, for example, be mentioned.

[0035] As the vector into which the expression cassette is integrated, a plasmid derived from *E. coli* such as pBR322, pBR325, pUC118, pUC119, pUC18, pUC19 or the like may suitably be used. The vector preferably has a marker to select the transformant. Such a marker may, for example, be *ura4* gene (an auxotrophic complementation marker) or isopropyl malate dehydrogenase gene (*leu1* gene).

[0036] In a case where an expression cassette containing the *SP-TF* gene is integrated into *S. pombe* chromosome by a homologous recombination method, the target site into which the expression cassette is integrated may be present in only one position of the S. *pombe* chromosome, or may be present in two or more positions. In a case where two or more target sites are present, the vector may be integrated into two or more positions in the *S. pombe* chromosome. When the expression cassette is integrated into one target site, the target site disclosed in JP-A-2000-262284 may, for example, be used. By using two or more types of vectors having different recombination regions, the vectors can be integrated into different target sites.

[0037] As the vector having an expression cassette containing the *SP-TF* gene integrated, a vector having a secretion signal peptide gene and a *hTF* gene integrated, disclosed in Patent Document 1, Patent Document 3 or the like may, for example, be used.

[0038] In the transformant of the present invention, Gas2 gene (SPBC29A10.08.1) endogenous to *S. pombe* as a host is deleted or inactivated. As shown in the following Examples, when a transformant of *S. pombe* having the *SP-TF* gene introduced is cultured at high density at a cell-density (optical density measured by a turbidimeter at 660 nm: OD_{660}) of at least 100, the expression amount of Gas2 (1,3-β-glucanosyl transferase) protein, as well as TF, increases. Gas2 protein is a glycoprotein localizing in the cell wall and involved in the biosynthesis of the cell wall of fission yeast, and is not considered to be directly involved in expression and secretion of TF. Nevertheless, in the high density cell culture process of TF expression strain (transformant having the *SP-TF* gene introduced), Gas2 protein as well as TF protein is secreted in a large amount into the liquid medium, and TF production efficiency decreases. Strangely, the increase of the expression and secretion amount of Gas protein observed at the time of high density culture of TF expression strain is not clearly observed at the time of common batch culture at OD_{660} of from 20 to 30. Since *Gas2* gene is deleted or inactivated in the transformant of the present invention, Gas2 protein is not expressed and the secretion amount of TF into the liquid medium increases, even at the time of the high density culture.

[0039] Further, the entire nucleotide sequence of the *S. pombe* chromosomes is stored and opened to the public in "*Schizosaccharomyces pombe* Gene DB (http://www.genedb.org/genedb/pombe/)" of Sanger Institute. The sequence data of *S. pombe* genes described in the present specification are available from the data base by searching with a gene name or a systematic name.

[0040] The transformant of the present invention is preferably one in which at least one type of protease gene (gene encoding protease) is deleted or inactivated. When the protease activity of at least one type of protease gene endogenous to *S. pombe* is deleted or inactivated in the transformant, TF protein production efficiency increases, and TF protein production amount more increases. The transformant of the present invention is preferably one in which at least one type of gene selected from the group consisting of a serine protease gene group (a group of genes encoding serine protease), an amino peptidase gene group (a group of genes encoding amino peptidase), a carboxypeptidase gene group (a group of genes encoding carboxypeptidase), and a dipeptidase gene group (a group of genes encoding dipeptidase) is deleted or inactivated.

[0041] The transformant of the present invention may be one in which only one type of protease gene is deleted or inactivated, or may be one in which two or more types of protease gene are deleted or inactivated. Among them, preferred is a transformant in which at least one type of gene selected from the group consisting of a metalloproteinase gene group (a group of genes encoding metalloproteinase), a serine protease gene group, a cysteine protease gene group (a group of genes encoding cysteine protease) and an aspartic protease gene group (a group of genes encoding aspartic protease) is deleted or inactivated, and also preferred is a transformant in which at least one type of gene selected from the group consisting of a metalloproteinase gene group and a serine protease gene group and at least one type of gene selected from the group consisting of a cysteine protease gene group and an aspartic protease gene group, are deleted or inactivated.

[0042] The four types of protease gene group of *S. pombe* may, for example, be as follows.

[0043] Metalloproteinase gene group: *cdb4*(SPAC23H4.09), *mas2*(SPBC18E5.12c), *pgp1*(SPCC1259.10), *ppp20*(SPAC4F10.02), *ppp22*(SPBC14C8.03), *ppp51*(SPAC22G7.01c), *ppp52*(SPBC18A7.01), *ppp53*(SPAP14E8.04).

[0044] Serine protease gene group: isp6(SPAC4A8.04), *ppp16*(SPBC1711.12), *psp3*(SPAC1006.01), *sxa2*(SPAC1296.03c).

[0045] Cysteine protease gene group: *ppp80*(SPAC19B12.08), *pca1*(SPCC1840.04), *cut1*(SPCC5E4.04), *gpi8*(SPCC11E10.02c).

[0046] Aspartic protease gene group: *sxa1*(SPAC26A3.01), *yps1*(SPCC1795. 09), *ppp81*(SPAC25B8.17).

[0047] The transformant of the present invention may be produced by deleting or inactivating *Gas2* gene of the transformant having an expression cassette containing the *SP-TF* gene introduced into *S. pombe* as a host, and may also be produced by introducing an expression cassette containing the *SP-TF* gene into *S. pombe* having *Gas2* gene preliminarily deleted or inactivated. By using as a host a *S. pombe* mutant strain in which at least one type of protease gene is deleted or inactivated, a transformant into which the *SP-TF* gene is introduced, in which *Gas2* gene is deleted or inactivated, and in which at least one type of protease gene is deleted or inactivated, is obtained. The mutant strain in which at least one type of protease gene endogenous to *S. pombe* is deleted or inactivated may, for example, be preferably a *S. pombe* mutant strain in which *psp3* gene, *isp6* gene, *ppp53* gene, *ppp16* gene, *ppp22* gene, *sxa2* gene, *ppp80* gene and *ppp20* gene are deleted or inactivated, such as A8 strain as disclosed in Patent Document 2.

[0048] Deletion or inactivation of *Gas2* gene, and deletion or inactivation of protease gene may be carried out by publicly known methods. Specifically, the Latour system (Nucleic Acids Res. (2006) 34: e11, and WO2007/063919) can be used to delete the gene. Further, the gene may be inactivated by introducing mutation into a part of the gene by a mutant screening method using mutagens (Koubo Bunshi Idengaku Jikken-Hou, 1996, Japan Scientific Societies Press), random mutations using PCR (PCR Methods Application, 1992, vol. 2, p. 28-33) and the like. Further, the portion at which a specific gene is deleted or inactivated may be an ORF (open reading frame) portion or may be an expression control sequence. Particularly preferred is deletion or inactivation by substituting the ORF portion of structural gene by a marker gene by means of PCR-mediated homologous recombination (Yeast, vol. 15, pages 943 to 951, 1998).

[0049] As the transformation method, any known transformation method may be used. The transformation method may, for example, be a conventional method like lithium acetate method, electroporation method, spheroplast method, glass-beads method or the like, and a method disclosed in JP-A-2005-198612. Further, a commercially available yeast transformation kit may be used.

[0050] The transformant of the present invention may be cultivated in the same manner as a natural yeast of the genus

Schizosaccharomyces.

[0051] As a liquid medium for culturing the transformant, a known yeast culture medium may be used so long as it contains a carbon source, a nitrogen source, an inorganic salt, etc. which a yeast of the genus *Schizosaccharomyces* can utilizes, and is capable of efficiently culturing a yeast of the genus *Schizosaccharomyces.* The liquid medium may be a natural medium or a synthetic medium.

[0052] The liquid medium may, for example, be MMA (Minimal medium with agar), SDC (Synthetic dextrose complete medium), TES (0.5% Bacto-yeast extract, 3% glucose, supplemented with uracil, leucine, histidine, lysine, adenine) or YES (0.5% Bacto-yeast extract, 3% glucose, supplemented with uracil, leucine, histidine, lysine, adenine), or YPD (1% Bacto-yeast extract, 2% Bactopeptone, 2% glucose).

[0053] The carbon source may, for example, be a sugar such as glucose, fructose or sucrose. The nitrogen source may, for example, be an inorganic acid or an ammonium salt of an inorganic acid, such as ammonia, ammonium chloride or ammonium acetate, peptone or casamino acid. The inorganic salt may, for example, be magnesium phosphate, magnesium sulfate or sodium chloride. Specifically, a nutrient medium such as YPD medium (M.D.Rose et al.,"Methods In Yeast Genetics", Cold Spring Harbor Labolatory Press (1990)), a minimal medium such as MB medium (K.Okazaki et al., Nucleic AcidsRes., 18, 6485-6489 (1990)) and the like may be used. Cultivation may be carried out by using a publicly known cultivation method for yeasts such as a shaking cultivation, a stirring cultivation.

[0054] The cultivation temperature is preferably from 23 to 37°C, more preferably from 30 to 32°C. Further, the cultivation time may be set appropriately.

[0055] Cultivation may be carried out by batch culture or continuous culture.

[Method for producing TF]

[0056] By culturing a transformant (TF expression strain) using *S. pombe* as a host and having *SP-TF* gene in a liquid medium, TF protein secreted into the liquid medium after culture can be obtained. As the liquid medium for culturing the TF expression strain and the culture method, a publicly known yeast culture medium may be used and a publicly known culture method may be employed, in the same manner as the culture of the transformant of the present invention.

[0057] TF protein is easily decomposed under an acidic condition. Accordingly, in production of TF, the pH of the liquid medium for the culture of the TF expression strain is preferably at least 5.5, more preferably from 5.5 to 6.5. The longer the culture time, the more the pH of the culture broth tends to decrease, and accordingly the pH is properly adjusted so that the pH of the culture broth is maintained to be within a range of from 5.5 to 6.5 at the time of the culture for TF production.

[0058] In order to further increase the TF protein secretory production amount, the culture time for culturing the TF expression strain is preferably at least 2 days, more preferably from 3 to 6 days, further preferably from 3 to 5 days.

[0059] The liquid medium for culturing the TF expression strain preferably contains adenine. In a case where the TF expression strain has the *SP-TF* gene with a copy number of at least one, lysis is likely to occur, and the TF expression amount tends to decrease. By culture in an adenine-containing liquid medium, even a TF expression strain having the *SP-TF* gene with a copy number of at least one favorably grows, thus increasing the TF secretory production efficiency. The adenine content in the liquid medium is, for example, from 0.5 to 200 mg/L, preferably from 1 to 100 mg/L per OD_{660} indicating the cell-density.

[0060] Further, in order to improve the TF productivity, the TF expression strain is preferably cultured at high density. It is particularly preferred to conduct fed-batch culture using an initial culture medium at a pH of from 5.5 to 6.5, preferably from 5.5 to 6.0 while the aeration rate into a culture tank, the stirring rate and the feeding rate of the feed medium are controlled. Since the medium is continuously added (fed) in accordance with the growth rate and the nutrition consumption rate while the glucose concentration in the medium is kept low at a certain level, the cell-density improves finally several tens times as compared with batch culture in a test tube or in a flask, a high cell-density is achieved, and a larger amount of TF can be produced by secretion. By high concentration culture of the TF expression strain, culture is conducted preferably until OD_{660} reaches 100 or higher, preferably OD_{660} reaches 200 to 800.

[0061] The TF expression strain is preferably the transformant of the present invention. In the transformant of the present invention, Gas2 protein derived from the host is not expressed, and the TF secretory production efficiency is high. In addition, at the time of purification of TF protein from the liquid medium after the high density culture, purification by isolation from Gas2 protein is not necessary, and purification can be carried out more easily.

[0062] TF protein can be obtained from the liquid medium after culture by a publicly known method. For example, cells are separated and removed from the liquid medium after culture by centrifugal separation, and the obtained culture supernatant is adsorbed e.g. in an affinity column, followed by washing and elution; impurities such as a dye are removed by activated carbon; or separation may be carried out by using a separation membrane. In a case where the TF expression strain having Gas2 gene is cultured at high density, since the recovered culture supernatant contains Gas2 protein in a large amount, after the culture supernatant is subjected to anion exchange chromatography using a DEAE resin, Gas2 protein is inevitably contained in a fraction containing TF protein. Accordingly, TF protein can be separated and purified from Gas2 protein only after the fraction containing TF protein is subjected to hydrophobic interaction chromatography or gel permeation chromatography. Whereas from the culture supernatant after the high density culture of the transformant of the present invention, TF protein can be purified only by subjecting the culture supernatant to anion exchange chromatography using a DEAE resin once.

EXAMPLES

[0063] Now, the present invention will be described in further detail with reference to Examples of the present invention and Comparative Examples. However, it should be understood that the present invention is by no means restricted to the following description. In the following Examples, "%" means "mass%" unless otherwise specified.

[EXAMPLE 1]

<Preparation of hTF mutant gene>

[0064] A natural *hTF* gene ORF (SEQ ID NO:1) encodes a protein with a full-length of 698 residues, having hTF protein fused to a downstream side of secretion signal peptide having 19 residues. By using human cDNA library as a template, PCR was carried out with a primary pair (forward primer No. 9220 (SEQ ID NO:3) and reverse primer No. 9221(SEQ ID NO:4)) to obtain a DNA fragment having restriction enzyme site *Afl*II and a nucleotide sequence encoding 4 residues (Leu-Lys-Lys-Arg) of the C-terminal portion of human PDI1 abx-domain at the 5' end of a gene encoding hTF protein, and restriction enzyme site *Xba*I respectively added at the 3' end, respectively.

[0065] A double digestion product with *Afl*II and *Xba*I of the DNA fragment obtained was integrated by ligation with a double digestion product with *Afl*II and *Xba*I of pSL6P3 (Patent Document 3) which is a chromosome single-locus integration type secretory expression vector of fission yeast having a gene encoding P3 secretion signal peptide downstream hCMV promoter, and having a multiple cloning site between the gene and LPI terminator, to transform *E. coli* DH5α to prepare plasmid DNA of expression vector pSL6P3-hTF having a gene encoding natural hTF protein containing 4 residues of the C-terminal portion of abx-domain at the N-terminal cloned.

[0066] From a secretory expression vector having an expression cassette of the desired protein integrated into the multiple cloning site of pSL6P3 vector, one copy of a linearized vector fragment cleaved by restriction enzyme *Not*I*,* is integrated between adjacent two gene loci *leu1-32* and *top2* on the fission yeast chromosome, by chromosome homologous recombination. Further, into pSL6P3 vector, a leucine marker gene fragment (*leu1*$^+$) to complement point mutation (*leu1-32*) portion of mutant (*leu1*$^-$) gene (a gene having leucine synthesis gene (*leu1*) inactivated by point mutation (*leu1-32*)) is integrated. Accordingly, in a case where a leucine-auxotrophic yeast containing mutant (*leu1*$^-$) gene is used as a host strain, the transformant having the linearized fragment of pSL6P3 vector integrated with the chromosome is no more auxotrophic for leucine, and the clone can be selected from the minimal medium (MMA plate) containing no leucine.

[0067] By PCR method, into the *hTF* gene in pSL6P3-hTF, a mutation to substitute 413rd and 611st (432nd and 630th of SEQ ID NO:1) aspartic residues of natural hTF protein with glutamic residue was introduced to prepare pSL6P3-hTF(N413Q/N611Q). Specifically, four point mutations to substitute the first nucleotide in the codon encoding each aspartic residue with cytosine (C) and the third nucleotide with adenine (A), were introduced. The sequence of hTF(N413Q/N611Q) having such mutations introduced is shown in SEQ ID NO: 2.

<Preparation of hTF(N413Q/N611Q) expression vector>

[0068] A DNA fragment containing a region encoding hTF(N413Q/N611Q) having pSL6P3-hTF(N413Q/N611Q) double-digested with *Afl*II and *Xba*I*,* was integrated by ligation with a double digestion product with *Afl*II and *Xba*I of chromosome single-locus integration type secretory expression vector pSL6PDI1(SP)Hsabx-*Afl*II of fission yeast having a gene encoding secretory carrier protein (PDI1(SP)-Hsabx) comprising a secretion signal peptide of S. *pombe* PDI1, ab-domain of human PDI1 and x-domain of S. *pombe* PDI1, downstream of hCMV promoter, and having a multiple cloning site between the gene and LPI terminator, to transform *E. coli* DH5α to prepare pSL6PDI1(SP)Hsabx-hTF(N413QN611Q) (8749bp, Fig. 1) which is a chromosome single-locus integration type expression vector of a fusion protein of secretory carrier protein (PDI1(SP)-Hsabx) and mutant hTF(N413Q/N611Q) protein.

<Preparation of A8 strain>

[0069] From leucine/uracil-auxotrophic (*leu1*$^-$ *ura4*$^-$) ARC010 strain (genotype: *h*$^-$ *leu1-32 ura4-D18*) of *S. pombe,* by Latour method, eight genes i.e. *psp3* gene, *isp6* gene, *ppp53* gene, *ppp16* gene, *ppp22* gene, *sxa2* gene, *ppp80* gene and *ppp20* gene were deleted to prepare A8 strain (genotype: *h*$^-$ *leu1-32 psp3-D13 isp6-D14 oma1-D10 ppp16-D20 fma2-D13 sxa2-D15 aap1-D17 ppp80-D11).* The A8 strain was a leucine-auxotrophic strain. More particularly, by the

Latour method, a gene deletion fragment (Latour fragment, comprising homologous recombination regions at both terminals, and *ura4*$^+$ marker gene and OL (overlapped sequence) region sandwiched between the homologous recombination regions), was once integrated by homologous recombination into the upstream or downstream side of the region to be deleted on the fission yeast genome, to prepare a latent strain. Then, the latent strain was cultured in a medium containing 5'-FOA to cause homologous recombination between OLs integrated, to form a gene deletion strain from which the region to be deleted containing *ura4*$^+$ marker gene dropped off, as a colony. Deletion of the desired gene from the obtained gene deletion strain was confirmed by PCR. By this method, since no foreign gene containing *ura4*$^+$ marker gene does not remain, only the desired gene can be deleted without integrating a foreign gene, and in addition, deletion of gene can be carried out repeatedly while the *ura4*$^+$ marker gene is recycled. The nucleotide sequence of the primer used for preparation of the Latour fragment to delete the genes by PCR is shown in Table 1.

TABLE 1

Gene	Primer	Nucleotide sequence	SEQ ID NO
psp3	Forward primer #6164	GCAAGTACTGCTCTTGATCTG	5
	Reverse primer #6167	TAAAGCATCCACTTTGAGCAA	6
isp6	Forward primer #6153	TCTGTTGGCAACATGTCTC	7
	Reverse primer #6156	GTGAAAGTAGATTATCCATGAGGTC	8
oma1	Forward primer #7243	TGCCTTTTACGGTTCTTTGGTC	9
	Reverse primer #7246	TGCACATCAGGTAGCCCGAC	10
ppp16	Forward primer #7255	TCATTAAGTCGTCGTCAAACGG	11
	Reverse primer #7258	CTTTAGGTCGTCAATTTCGTGC	12
fma2	Forward primer #6917	CATACTCCCCTGATGTTTCGC	13
	Reverse primer #6920	CATTCATGGTGGTAAGAGTGTTCC	14
sxa2	Forward primer #9515	ACCAATCTTCCATATCCCCTTG	15
	Reverse primer #9518	GCTACGAAAGCTTCAACTGCG	16
aap1	Forward primer #9883	AAAGTTCTTTGTTTTCAATTTCCATG	17
	Reverse primer #9886	TCCTTATAAACGAGCTATCGAATTG	18
atg4	Forward primer #6175	GAATCATACTACGATCAGTTGC	19
	Reverse primer #6178	GAAATTTACTCACAGGGAAGAAC	20

<Preparation of A8-gas2Δ strain >

[0070] From the A8 strain, *Gas2* gene was deleted by Latour method to prepare A8-gas2A strain (genotype: *h$^-$ leu1-32 psp3-D13 isp6-D14 oma1-D10 ppp16-D20 fma2-D13 sxa2-D15 aap1-D17 ppp80-D11 gas2-D15).* Deletion of the desired gene from the obtained gene deletion strain was confirmed by PCR. The A8-gas2Δ strain was a leucine-auxotrophic strain. The nucleotide sequence of the primer used for preparation of the Latour fragment to delete Gas2 gene by PCR is shown in Table 2.

TABLE 2

Gene	Primer	Nucleotide sequence	SEQ ID NO
gas2	Forward primer #10493	GAATGTCCATATAAGTCAATGATGAGC	21
	Reverse primer #10496	GAGGTTAACGACTATGGTCTTGTTAAC	22

[0071] The prepared A8 strain and A8-gas2Δ strain were subjected to growth evaluation (measurement of μmax and growth curve) on a test tube scale (under conditions of inoculation to 5mL of YES and culture at 30°C for 68 hours). As a result, both the strains were confirmed as follows. Although the maximum relative growth rate (μmax relative value) indicating the initial rising of cell growth slightly decreased (a decrease of about 8 to 12%) as compared with the same auxotrophic (leu1$^-$) wild type strain (ARC001), the ultimate cell-density (OD_{660}) slightly increased (an increase of about

9 to 17%), and both the strains could grow in the same manner as leucine-auxotrophic wild type strain (not shown). Particularly, no difference in the growth and the phenotype between A8 strain and A8-gas2Δ strain was observed.

<Preparation of hTF(N413Q/N611Q) gene one copy expression strain>

[0072] pSL6PDI1(SP)Hsabx-hTF(N413QN611Q) was cleaved by restriction enzyme *Not*I to prepare a linearized vector fragment (6993 bp), and the vector fragment was integrated by means of lithium acetate method to transform leucine-auxotrophic *S. pombe* A8 strain of A8-gas2Δ strain as a host. Cells after transformation were applied to a MMA plate, and a colony formed by being made to be leucine-auxotrophic, was obtained as a recombinant clone. Then, a clone which was confirmed by PCR that the desired gene was appropriately introduced, was selected as a positive clone (hTF(N413Q/N611Q) expression strain). Transformants having pSL6PDI1(SP)Hsabx-hTF(N413QN611Q) introduced into A8 strain and A8-gas2Δ were respectively named A8-hTF(1) strain and A8-gas2Δ-hTF(1) strain.

<Secretory production of hTF(N413Q/N611Q) one copy expression strain>

[0073] A8-hTF(1) strain and A8-gas2Δ-hTF(1) strain were cultured to express hTF(N413Q/N611Q) by secretion. Specifically, in 5mL of YES in a glass test tube, they were subjected to raising culture and pre-culture respectively for 24 hours, and in 5mL of YPD+MES medium(a liquid medium having 0.3M MES buffer contained in YPD) (pH 6.0) in a glass test tube, shake-cultured at 32°C for 3 days or 5 days. The cell-density at the completion of culture (ultimate OD_{660}) was from 30 to 36. The culture supernatant fraction recovered from the culture broth and the cell pellet were respectively subjected to SDS-PAGE analysis and confirmed that hTF(N413Q/N611Q) were expressed inside and outside the cells (not shown). Further, with respect to both A8-hTF(1) strain and A8-gas2Δ-hTF(1) strain, the hTF(N413Q/N611Q) amount in the cell pellet was larger and the hTF(N413Q/N611Q) amount in the culture supernatant was larger in the one cultured for 5 days than in the one cultured for 3 days. Therefore, it was conformed that by prolonging the culture time, the secretion amount of hTF(N413Q/N611Q) can be increased and the amount remaining in the cells without secretion can be decreased.

<Results of hTF(N413Q/N611Q) one copy expression strain>

[0074] A8-gas2Δ-hTF(1) strain was cultured to express hTF(N413Q/N611Q) by secretion. Specifically, in 5 mL of YES in a glass test tube, the strain was subjected to raising culture and pre-culture respectively for 24 hours, and then using a 24-well plate, shake-cultured in 5 mL of an adenine-containing YPD+MES medium (pH 6.0) at 32°C for 3 days, 4 days or 5 days, and subjected to ELISA using a commercial kit (tradename: "Human Transferrin ELISA Quantitation Set", manufactured by Bethyl Laboratories), and the hTF(N413Q/N611Q) protein amount secreted into the culture supernatant after completion of the culture was measured. The longer the culture time, the larger the hTF(N413Q/N611Q) secretion amount. The secretion amount of hTF(N413Q/N611Q) per culture supernatant over 5 days of culture was from 30 to 40 mg/L in A8-gas2Δ-hTF(1) strain.

<High density culture of hTF(N413Q/N611Q) one copy expression strain>

[0075] A8-hTF(1) strain and A8-gas2Δ-hTF(1) strain were cultured at high density to express hTF(N413Q/N611Q) by secretion. Specifically, on a 5L culture scale, fed-batch culture was conducted using an initial culture medium at a pH of the culture broth of from 5.5 to 6.0 while the aeration rate into a culture tank, the stirring rate and the feeding rate of the feed medium were controlled. As the liquid medium, an adenine-containing semisynthetic medium (several % Bacto-yeast extract, synthetic components such as glucose, vitamins, minerals, inorganic salts, etc.) was used. The medium was continuously added (fed) in accordance with the growth rate and the nutrition consumption rate, while the glucose concentration was kept low at 2%. Finally, the cell-density (OD_{660}) reached a level of from 200 to 800.

[0076] The culture broth was sampled with time and subjected to SDS-PAGE, and the results are shown in Fig. 2. 5 μL of the culture broth was applied to each lane. In Fig. 2, "hTF" indicates a hTF(N413Q/N611Q) band. As shown in the CBB staining image in Fig. 2, expression of gas2 was suppressed in A8-gas2Δ-hTF(1) strain, and the secretion amount of hTF(N413Q/N611Q) in A8-gas2Δ-hTF(1) strain was larger than in A8-hTF(1) strain in all the cases of the culture time of 48, 72 and 96 hours.

[0077] Further, ELISA was conducted in the same manner as above using the sampled culture broth, and the hTF(N413Q/N611Q) protein amount secreted into the culture supernatant was measured. As shown in Fig. 3, there was no difference in the secretion amount between the two strains from beginning of the culture up to about 48 hours, however, after about 48 hours, the secretion amount of hTF(N413Q/N611Q) in A8-gas2Δ-hTF(1) strain was definitely larger than in A8-hTF(1) strain. The ultimate secretion amounts of the two strains (secretion amounts 168 hours after beginning of the culture in the case of A8-hTF(1) strain and 96 hours after beginning of the culture in the case of A8-

gas2Δ-hTF(1) strain) are shown in Table 3. Of A8-gas2Δ-hTF(1) strain, the hTF secretion amount per culture broth was at least 4.8 times and the hTF secretion amount per cell (per OD_{660}) was at least 2.1 times those of A8-hTF(1) strain.

TABLE 3

hTF expression	hTF secretion amount per culture broth (g/L)	hTF secretion amount per cell (mg/OD660)
A8-hTF(1) strain	0.09	2.04
A8-gas2Δ-hTF(1) strain	0.43	4.33

[0078] The cells were removed by centrifugal separation from the culture broth after high density culture, and the recovered culture supernatant was subjected to DEAE chromatography to purify hTF. As the DEAE column, a column packed with a DEAE resin (DEAE Sepharose FF(Fast Flow)) (tradename: "XK26", manufactured by GE Healthcare) was used. Specifically, 900 mL of the culture supernatant was applied to a DEAE column (1 CV=60.6 mL) equilibrated with an equilibration buffer (20 mM Tris-HCl, pH 8.5), and then an elution buffer B (20 mM Tris-HCl, 1M NaCl, pH 8.5) was applied under gradient conditions of from 0% (15 CV) to 100% (5 CV) for elution. The fraction size was 30.3 mL.

[0079] Fractions containing hTF(N413Q/N611Q) protein (the 3rd to 6th fractions) were recovered together and subjected to SDS-PAGE, and the results are shown in Fig. 4. In Fig. 4, the lane "after deletion of Gas2" indicates the CBB staining image of the purified fractions from the culture broth of A8-gas2Δ-hTF(1) strain, and the lane "before deletion of Gas2" indicates the CBB staining image of the purified fractions from the culture broth of A8-hTF(1) strain. As shown in the CBB staining images in Fig. 4, in the purified fraction from the culture broth of A8-gas2Δ-hTF(1) strain, only a hTF(N413Q/N611Q) protein band was detected, and it was confirmed that hTF(N413Q/N611Q) protein could be separated from other proteins and purified at high purity only by DEAE chromatography once. Whereas in the purified fraction from the culture broth of A8-hTF(1) strain, in addition to the hTF(N413Q/N611Q) protein band, a plurality of bands were detected at higher molecular weight side. Among them, the band in the vicinity of 115 kDa was taken out and subjected to N terminal sequencing analysis and confirmed to be a Gas2 band. Since secretion of Gas2 was not definitely confirmed by culture at conventional cell-density, it was considered to be characteristic of high density culture. In A8-hTF(1) strain, Gas1 secretory production amount increases in addition to hTF(N413Q/N611Q), which indicates that the hTF(N413Q/N611Q) secretion production amount is kept low as compared with A8-gas2Δ-hTF(1) strain.

[0080] The entire disclosure of Japanese Patent Application No. 2016-003606 filed on January 12, 2016 including specification, claims, drawings and summary is incorporated herein by reference in its entirety. (

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Asp Gly Pro Ser Val Ala Cys Val Lys Lys Ala Ser Tyr Leu Asp Cys
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Ile Arg Ala Ile Ala Ala Asn Glu Ala Asp Ala Val Thr Leu Asp Ala
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GTG GCA GAG TTC TAT GGG TCA AAA GAG GAT CCA CAG ACT TTC TAT TAT    288
Val Ala Glu Phe Tyr Gly Ser Lys Glu Asp Pro Gln Thr Phe Tyr Tyr
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Ala Val Ala Val Val Lys Lys Asp Ser Gly Phe Gln Met Asn Gln Leu
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Pro Leu Glu Lys Ala Val Ala Asn Phe Phe Ser Gly Ser Cys Ala Pro
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Cys Ala Asp Gly Thr Asp Phe Pro Gln Leu Cys Gln Leu Cys Pro Gly
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Leu Lys Cys Asp Glu Trp Ser Val Asn Ser Val Gly Lys Ile Glu Cys
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GTA TCA GCA GAG ACC ACC GAA GAC TGC ATC GCC AAG ATC ATG AAT GGA   1152
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Lys Cys Gly Leu Val Pro Val Leu Ala Glu Asn Tyr Gln Lys Ser Asp
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AAT TGT GAG GAT ACA CCA GAG GCA GGG TAT TTT GCT GTA GCA GTG GTG   1296
Asn Cys Glu Asp Thr Pro Glu Ala Gly Tyr Phe Ala Val Ala Val Val
            420                 425                 430
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Lys Asn Pro Asp Pro Trp Ala Lys Asn Leu Asn Glu Lys Asp Tyr Glu
545                 550                 555                 560
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Cys His Leu Ala Arg Ala Pro Asn His Ala Val Val Thr Arg Lys Asp
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Lys Glu Ala Cys Val His Lys Ile Leu Arg Gln Gln Gln His Leu Phe
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Gly Ser Gln Val Thr Asp Cys Ser Gly Asn Phe Cys Leu Phe Arg Ser
    610                 615                 620
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Glu Thr Lys Asp Leu Leu Phe Arg Asp Asp Thr Val Cys Leu Ala Lys
625                 630                 635                 640
CTT CAT GAC AGA AAC ACA TAT GAA AAA TAC TTA GGA GAA GAA TAT GTC  1968
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Claims

1. A transformant using *Schizosaccharomyces pombe* as a host, comprising a transferrin gene and a secretion signal peptide gene expressing transferrin to which a secretion signal peptide which functions in the host binds, located in an upstream region of the transferrin gene, and having Gas2 gene endogenous to the host deleted or inactivated.

2. The transformant according to Claim 1, wherein the transferrin gene is a gene encoding natural TF protein derived from a mammal, or its modified protein.

3. The transformant according to Claim 1 or 2, wherein the transferrin gene is a gene encoding human transferrin or its modified protein.

4. The transformant according to Claim 1 or 2, wherein the transferrin gene is a mutant transferrin gene having a mutation introduced to a gene encoding natural mammal transferrin, and the mutant transferrin gene is a gene encoding mutant transferrin having at least one aspartic residue which is a N-linked glycosylated portion of natural transferrin deleted or substituted with another amino acid residue.

5. The transformant according to Claim 4, wherein the transferrin gene is a mutant transferrin gene having a mutation introduced to a gene encoding natural human transferrin.

6. The transformant according to any one of Claims 1 to 5, wherein at least one protease gene endogenous to the host is deleted or inactivated.

7. The transformant according to Claim 6, wherein the protease gene is a gene selected from the group consisting of a metalloproteinase gene group, a serine protease gene group, a cysteine protease gene group and an aspartic protease gene group.

8. The transformant according to Claim 6 or 7, wherein the protease gene is a gene selected from the group consisting of psp3 gene, isp6 gene, ppp53 gene, ppp16 gene, ppp22 gene, sxa2 gene, ppp80 gene and ppp20 gene.

9. The transformant according to any one of Claims 1 to 8, wherein the transferrin gene is located downstream of a gene encoding a secretory carrier protein containing a secretion signal peptide, directly or indirectly.

10. The transformant according Claim 9, wherein the gene encoding a secretory carrier protein is a fusion gene of a gene encoding a secretion signal peptide portion of host PDI1, a gene encoding an ab-domain portion of human PDI1, and a gene encoding an x-domain portion of host PDI1.

11. A method for producing transferrin, which comprises culturing the transformant as defined in any one of Claims 1 to 10 in a liquid medium and obtaining transferrin from the liquid medium.

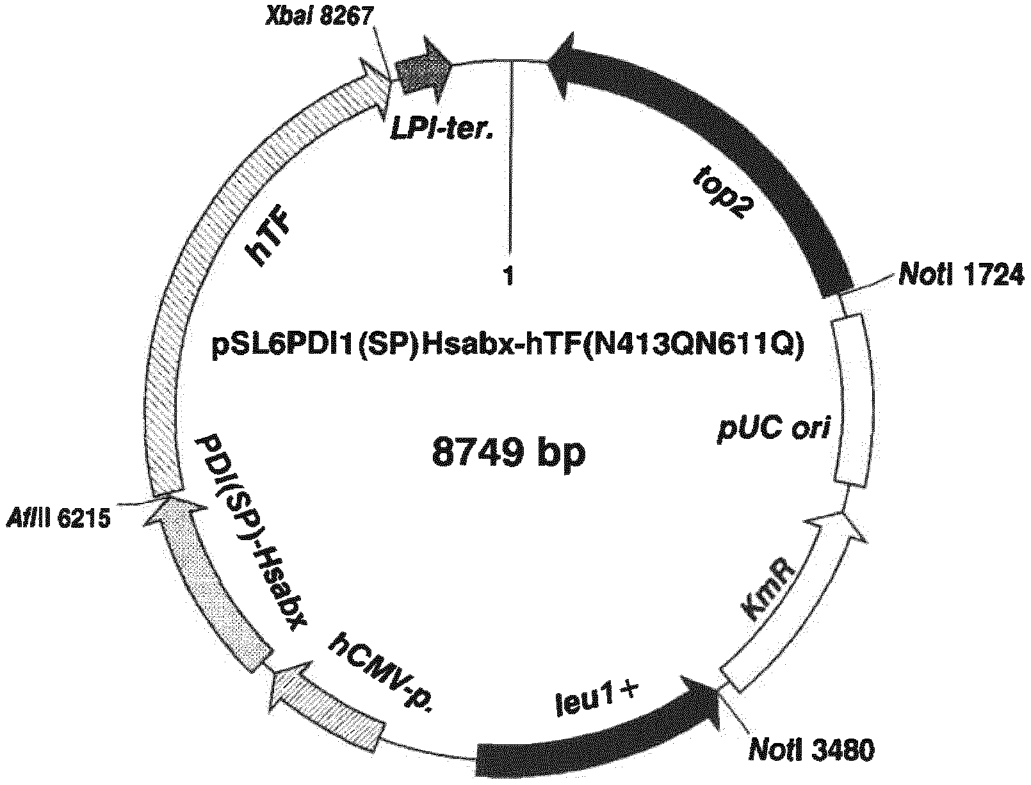
12. The method for producing transferrin according to Claim 11, wherein the transformant is cultured in a liquid medium having a pH of from 5.5 to 6.5.

13. The method for producing transferrin according to Claim 11 or 12, wherein the transformant is cultured in a liquid medium containing adenine.

14. The method for producing transferrin according to any one of Claims 11 to 13, wherein the transformant is cultured until the cell-density (OD_{660}) reaches 100 or higher, and then transferrin is obtained from the liquid medium.

15. A method for producing a transformant, which comprises integrating into *Schizosaccharomyces pombe* as a host, a secretion signal peptide gene which functions in the host, and a transferrin gene located downstream of the secretion signal peptide gene, and deleting or inactivating Gas2 gene endogenous to the host.

Fig. 1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LPI-ter.
top2
NotI 1724
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8749 bp
pUC ori
KmR
NotI 3480
leu1+
hCMV-p.
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AflII 6215
hTF

Fig. 2

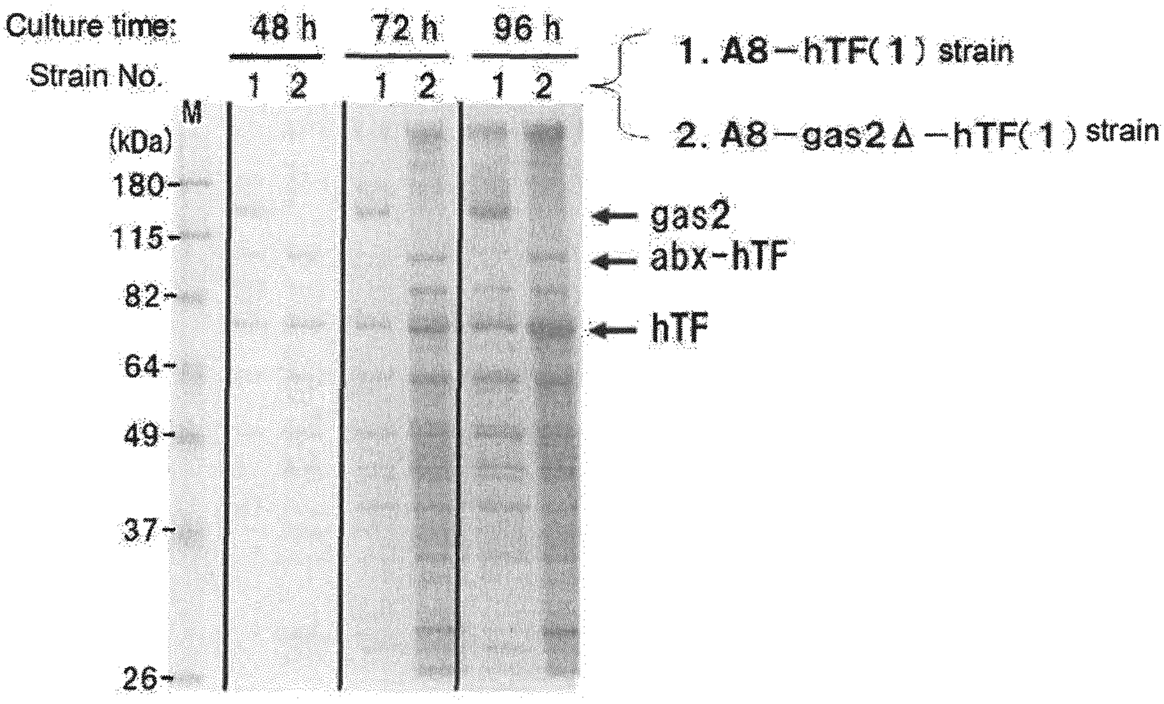
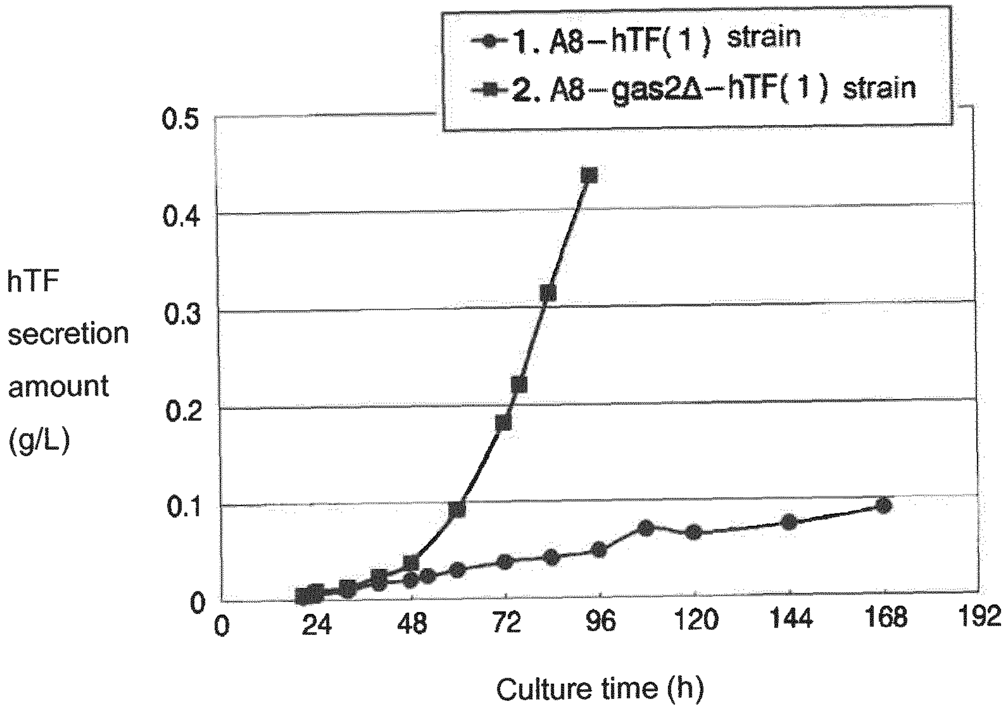

Culture time:
48 h
72 h
96 h
Strain No.
1 2
1 2
1 2
1. A8−hTF(1) strain
2. A8−gas2Δ−hTF(1) strain
M
(kDa)
180
115
82
64
49
37
26
gas2
abx-hTF
hTF

Fig. 3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2. A8−gas2Δ−hTF(1) strain
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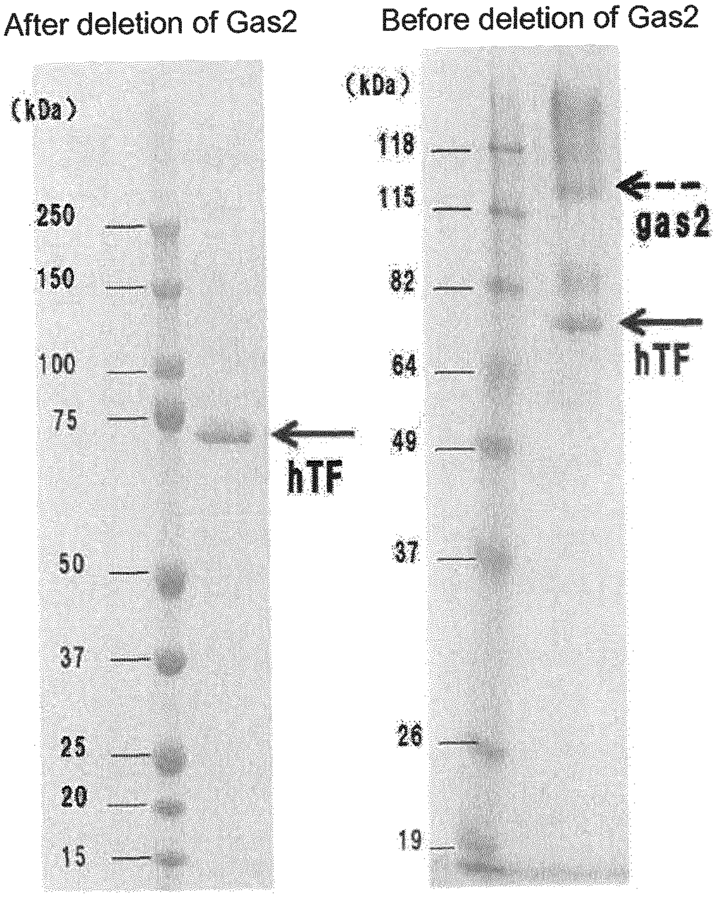
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
144
168
192
Culture time (h)

Fig. 4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(kDa)
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150
100
75
50
37
25
20
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hTF
Before deletion of Gas2
(kDa)
118
115
82
64
49
37
26
19
gas2
hTF

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2017/000497

A. CLASSIFICATION OF SUBJECT MATTER

C12N15/09(2006.01)i, *C12N1/19*(2006.01)i, *C12P21/00*(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N15/09, C12N1/19, C12P21/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho	1922-1996	Jitsuyo Shinan Toroku Koho	1996-2017
Kokai Jitsuyo Shinan Koho	1971-2017	Toroku Jitsuyo Shinan Koho	1994-2017

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580(JDreamIII), CAplus/MEDLINE/EMBASE/BIOSIS(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007/015470 A1 (Asahi Glass Co., Ltd.), 08 February 2007 (08.02.2007), claims; paragraphs [0009], [0010] & US 2008/0206816 A1 claims; paragraphs [0021], [0022] & US 2013/0122547 A1 & EP 1930428 A1 & EP 2182060 A1 & CA 2617832 A & JP 2007-529263 A & JP 2010-220626 A & JP 5131457 B2 & JP 513493 B2	1-15
A	JP 2011-125281 A (Asahi Glass Co., Ltd.), 30 June 2011 (30.06.2011), claims (Family: none)	1-15

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 16 March 2017 (16.03.17)	Date of mailing of the international search report 04 April 2017 (04.04.17)
Name and mailing address of the ISA/ Japan Patent Office 3-4-3,Kasumigaseki,Chiyoda-ku, Tokyo 100-8915,Japan	Authorized officer Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2017/000497

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE MEDINA-REDONDO, M. et al., β(1,3)-Glucanosyl-Transferase Activity Is Essential for Cell Wall Integrity and Viability of Scyzosaccharomyces pombe, PLoS ONE, 2010, Vol.5, e14046	1-15

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- WO 2007015470 A **[0006]**
- WO 2013111754 A **[0006]**
- WO 199623890 A **[0027]**
- JP 2776085 B **[0032]**
- JP H07163373 A **[0032]**
- JP H10215867 A **[0032]**
- JP H11192094 A **[0032]**
- JP 2000262284 A **[0032] [0036]**
- WO 96023890 A **[0032]**
- WO 9923223 A **[0034]**
- WO 200726617 A **[0034]**
- WO 2014030644 A **[0034]**
- WO 2007063919 A **[0048]**
- JP 2005198612 A **[0049]**
- JP 2016003606 A **[0080]**

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- Koubo Bunshi Idengaku Jikken-Hou. Japan Scientific Societies Press, 1996 **[0048]**
- *PCR Methods Application,* 1992, vol. 2, 28-33 **[0048]**
- *Yeast,* 1998, vol. 15, 943-951 **[0048]**
- **M.D.ROSE et al.** Methods In Yeast Genetics. Cold Spring Harbor Labolatory Press, 1990 **[0053]**
- **K.OKAZAKI et al.** *Nucleic AcidsRes.,* 1990, vol. 18, 6485-6489 **[0053]**